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Changes in Intracellular Cytokine Levels in Newborn and Adult Lymphocytes Induced by HSV-1

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Changes in the expression of intracellular interleukin-2 (IL-2), interleukin-4 (IL-4), interferon (IFN)-γ, and tumor necrosis factor (TNF)-α in newborn and adult lymphocytes induced by herpes simplex virus (HSV)-1 were examined. Cord blood mononuclear cells (CBMC) or adult peripheral blood mononuclear cells (PBMC) were infected with HSV-1 and cultured with phorbol 2-myristate 13-acetate (PMA) plus ionomycin in the presence of monensin for 4 hr. Surface antigen and intracellular cytokines were stained simultaneously and analyzed by flow cytometry. The percentage of cells that expressed IL-2, IFN-γ, and TNF-α was significantly increased in HSV-1-infected $CD3^+$, $CD4^+$, $CD8^+$, $CD45RA^+$, and $CD45R0^+$ lymphocytes compared with uninfected lymphocytes from adult PBMC. The percentage of cells that expressed IL-2 and TNF-α was increased significantly in HSV-1-infected $CD3^+$, $CD4^+$, $CD8^+$, and $CD45RA^+$ lymphocytes compared with uninfected lymphocytes from CBMC. IFN-γ was under the detectable level in HSV-1-infected and uninfected lymphocytes from CBMC. Intracellular IL-4 was not detected in HSV-1 or in uninfected lymphocytes from PBMC and CBMC. These results demonstrate that HSV-1 enhances intracellular levels of IL-2, IFN-γ, and TNF-α in adult lymphocytes and defective IFN-γ production in cord blood. ***J. Med. Virol. 56:145–150, 1998.*** © 1998 Wiley-Liss, Inc.

KEY WORDS: intracellular cytokine; interleukin-2; interferon-γ; tumor necrosis factor-α; HSV-1

INTRODUCTION

Herpes simplex virus (HSV) causes severe infection with a high mortality in neonates [Whitley et al., 1991]. Immaturity of natural killer (NK) cells and a low frequency of specific T lymphocytes responding to HSV at birth are thought to be mechanisms of the severity of primary HSV infection in neonates [Hayward et al., 1984; Kohl et al., 1984; Leibson et al., 1986]. Cytokines are classified into two distinct subsets, type 1 [interleukin-2 (IL-2) and interferon-γ (IFN-γ)] and type 2 (IL-4, IL-5, IL-6, and IL-10) [Mosmann et al., 1986]. These cytokines play important roles in the activation and regulation of T lymphocytes in the immune system. HSV infection may alter cytokine production and affect the immune function of lymphocytes.

Kuo et al. [1993] reported that during acute HSV-1 infections, IL-2 production of HSV-1 infected cells in response to phytohaemagglutinine (PHA) was lower than that of uninfected cells. Usually, the production of cytokines is evaluated by measuring cytokines in body fluid or in the supernatant of cultured cells by bioassay or immunoassay. These methods do not permit the analysis of the frequency of phenotype of cytokine-producing cells [Hayward et al., 1988]. Intracellular cytokines can now be detected at the single-cell level by flow cytometry [Jung et al., 1993; Prussin and Metcalfe, 1995]. We investigated changes in intracellular cytokine levels induced by HSV-1 in a lymphocyte subpopulation and compared intracellular cytokine levels of lymphocytes from adults with those from newborns.

MATERIALS AND METHODS

Virus

HSV-1 (KOS strain) was grown at a multiplicity of infection (moi) of 0.1 on Vero cells that were cultured in minimum essential medium (MEM; Life Technologies Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS; Life Technologies Gibco BRL) at 37°C. After 48 hr, the supernatant was collected and cell debris was removed by centrifugation. The supernatant virus stock was stored at –70°C. The titer of HSV-1 in the virus stock was 2×10^6PFU/ml. Supernatant of uninfected Vero cells followed by sonication was collected to prepare the control solutions for HSV-1 preparations. In some experiments, virus solution was exposed to 56°C or UV light for 30 min for inactivation of virus before use.

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Preparation of Cord Blood Mononuclear Cells (CBMC), Peripheral Blood Mononuclear Cells (PBMC), and CD4-Rich Lymphocytes

Cord blood was obtained from the placental end of the cord at full-term birth. PBMC were separated from heparinized venous blood of healthy HSV-1 seropositive adults by Ficoll-Hypaque (Histopaque 1077, Sigma, St. Louis, MO) gradient centrifugation. CBMC and PBMC at the interface were collected and washed three times with RPMI-1640 (Gibco BRL). CD4-rich lymphocytes were obtained by the use of DYNABEADS M-450 CD4 (Dynal, Skoyøn, Norway). Briefly, 1×10^7 CBMC or PBMC and 75 μl of M-450 CD4 beads were mixed (bead to cell ration of 3:1) on ice for 30 min, 5 ml of phosphate-buffered saline (PBS) containing 1% FBS was added, and the beads were washed three times by the use of a magnetic particle concentrator (MPC). Rosette cells with beads were collected with MPC and detached by mixing 10 μl of DETACHaBEAD (Dynal) for 1 hr. Cells collected by removal of the detached beads with MPC were used for the CD4-rich population. This procedure resulted in greater than 95% $CD4^+$ cells. CBMC, PBMC or $CD4^+$ lymphocytes were suspended at a concentration of 1×10^6/ml in RPMI-1640 with 10% FBS.

HSV-1 Infection and Cell Culture

CBMC (1×10^6), PBMC (1×10^6), and $CD4^+$ lymphocytes (1×10^6) were centrifuged at 1500 rpm for 5 min. The pelleted cells were resuspended in HSV-1 stock at a moi of 1.0 or with control supernatant, then adsorbed for 90 min at 37°C with occasional mixing. The cells were washed three times with RPMI-1640 to remove unadsorbed virus and resuspended in a 1.0 ml volume (1×10^6 cells) in RPMI-1640 with 10% FBS in plastic tubes. Cells were immediately stimulated with 5 μg/ml phytohaemagglutinin (PHA, Sigma) or 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma) plus 1 μM ionomycin (Sigma). Monensin is known to inhibit the subcellular transport of secretary proteins without altering cell surface markers, thereby increasing the quantity of intracellular cytokines [Tartakoff, 1983]. Thus, 2 μM monensin (Sigma) was added to the culture for detection of intracellular cytokines.

Collection of Supernatant

HSV-1-infected and uninfected CBMC or PBMC were cultured at 37°C in 5% CO_2 for 24 hr with PMA plus ionomycin. Tubes were centrifuged at 1500 rpm for 5 min, and the supernatant was collected and stored at –30°C. The concentrations of cytokines in the supernatant were assayed using IL-2, IFN-γ, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN).

Analysis of HSV-1 Antigen by Flow Cytometry

Cells were washed with PBS and then fixed with 75% ethanol at –20°C for 20 min. They were then washed three times with PBS and resuspended in 100 μl of PBS. Next, 10 μl of fluorescein isothiocyanate (FITC)-conjugated rabbit anti-HSV-1 (Dako, Kyoto, Japan) was added and incubated for 1 hr at 37°C. The cells were washed three times with PBS. Immunofluorescence-stained cells were analyzed by flow cytometry.

Monoclonal Antibodies

The following monoclonal antibodies (MoAb) were used for staining surface antigen and intracellular cytokines: Per CP-conjugated mouse anti-Leu4 (CD3) MoAb, anti-Leu3a (CD4) MoAb, anti-Leu2a (CD8) MoAb, FITC-conjugated anti-CD45RA MoAb, phycoerythrin (PE)-conjugated anti-CD45RO MoAb (Becton Dickinson, San Jose, CA) and PE-conjugated anti-CD45RA MoAb, FITC-conjugated anti-CD45RO MoAb (Dako, Japan), FITC- or PE-conjugated anti-human interleukin-2 (Anti-Hu-IL-2) MoAb (clone 5344.111, mouse IgG1), FITC-conjugated anti-human interferon γ (Anti-Hu-IFN-γ) MoAb (clone 25723.11 mouse IgG2b), PE-conjugated anti-human tumor necrosis factor (TNF)-α (Anti-Hu-TNF-α, clone Mab11, mouse IgG1), PE-conjugated mouse anti-human IL-4 MoAb (clone 3010.211, mouse IgG1), FITC-conjugated mouse IgG2a (clone X40), PE-conjugated mouse IgG1 (clone X39) (Becton Dickinson).

Analysis of Intracellular Cytokines by Flow Cytometry

Intracellular cytokines of uninfected and HSV-1-infected cells were analyzed after 4 hr culture. One million cells were incubated with PerCP-conjugated anti-CD3, CD4, or CD8 MoAb, PE-conjugated anti-CD45RO MoAb or anti-CD45RA MoAb, and incubated at 4°C for 30 min. Cells were washed once and mixed with FACS permeabilizing solution (Becton Dickinson) and incubated for 10 min at room temperature in the dark. After washing, anti-cytokine antibodies were added and incubated for 30 min at room temperature in the dark. Cells were washed twice with cell wash and analyzed on a FACScan by use of the CELLQuest program.

Statistical Analysis

Wilcoxon signed rank test was used for analyzing the samples.

RESULTS

Levels of Intracellular Cytokines in Uninfected and HSV-1-Infected Cells

Uninfected and HSV-1-infected PBMC were stimulated with PHA in the presence or absence of monensin for 4 hr. The percentage of cells expressing IL-2, IFN-γ, and TNF-α among uninfected and HSV-infected $CD3^+$ lymphocytes in the presence or absence of monensin was less than 2%.

Uninfected and HSV-1-infected PBMC were cultured with PMA plus ionomycin in the presence or absence of monensin for 4 hr. A representative three-color analysis of cells from one normal adult is shown in Fig. 1. $CD3^+$ cells were gated and the histogram showed the

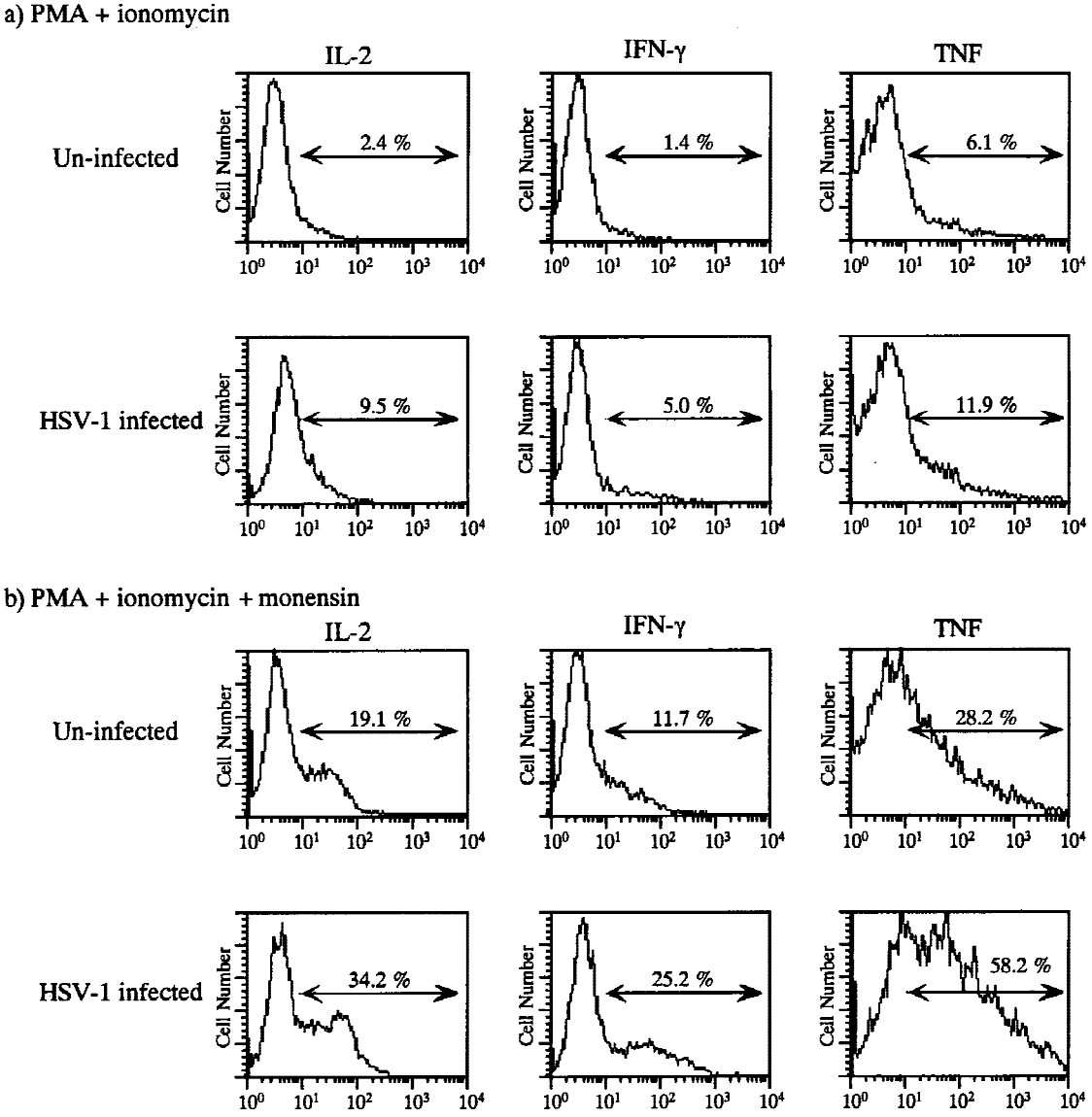

Fig. 1. Representative histogram of intracellular interleukin-2 (IL-2), interferon (IFN)-γ and tumor necrosis factor (TNF)-α from adult peripheral blood mononuclear cells (PBMC). Herpes simplex (HSV)-1-infected and uninfected PBMC were cultured for 4 h with phorbol 12-myristate 13-acetate (PMA) plus ionomycin in the presence or absence of monensin. **a:** PMA plus ionomycin. **b:** PMA plus ionomycin and monensin. PBMC were stained with PerCP-labeled anti-Leu4 (CD3) monoclonal antibody (MoAb), PE-labeled anti-IL-2 MoAb, or TNF-α MoAb and FITC-labeled anti-IFN-γ MoAb. $CD3^+$ cells were gated and the histogram of intracellular IL-2, IFN-γ, and TNF-α in $CD3^+$ cells is shown.

percentage of cells expressing IL-2, IFN-γ, and TNF-α among uninfected and HSV-1-infected $CD3^+$ cells. The percentage of cells that expressed IL-2, IFN-γ, and TNF-α was increased after culture with PMA plus ionomycin in the presence of monensin compared with that for culture in the absence of monensin. The percentage of cells that expressed IL-2, IFN-γ, and TNF-α in HSV-1-infected $CD3^+$ lymphocytes was greater than that in uninfected $CD3^+$ lymphocytes after culture with PMA plus ionomycin in the presence of monensin. The percentage of cells that expressed IL-4 was less than 2% in uninfected and HSV-1-infected $CD3^+$ lymphocytes after culture with PMA plus ionomycin in the absence or the presence of monensin (data not shown). The intracellular levels of IL-2, IFN-γ, and TNF-α in $CD3^+$ lymphocytes did not differ between 4, 8, and 12 hr cultures with PMA plus ionomycin in the presence of monensin (data not shown). A 4-hr culture assay was used for detection of intracellular cytokines in the following experiments.

The percentage of cells that expressed IL-2, IFN-γ, and TNF-α among HSV-1-infected $CD3^+$, $CD4^+$, $CD8^+$, $CD45RO^+$, and $CD45RA^+$ lymphocytes from adults was significantly greater than among uninfected cells (Fig. 2). The percentage of cells that expressed IL-2 among HSV-1-infected $CD4^+$ lymphocytes was significantly greater than that of HSV-1-infected $CD8^+$ lymphocytes from adults. The percentage of cells that expressed IFN-γ among HSV-1-infected $CD8^+$ lymphocytes was significantly greater than that of HSV-1-infected $CD4^+$

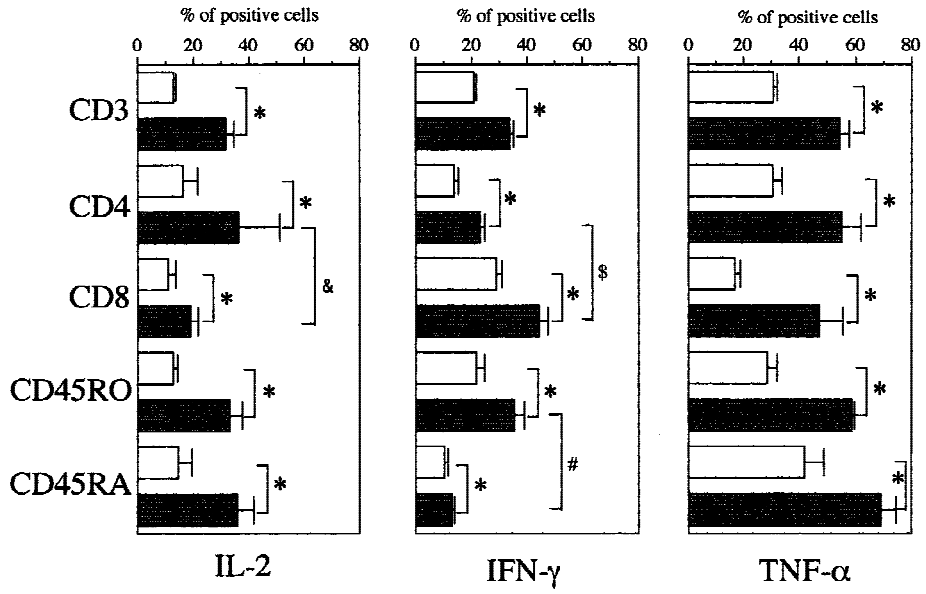

Fig. 2. Expression of intracellular IL-2, IFN-γ, and TNF-α in each lymphocyte subpopulation from normal adult peripheral blood mononuclear cells (PBMC). Data represent mean percentages of cells that expressed IL-2, IFN-γ and TNF-α in HSV-1-infected (shaded bars) and uninfected (open bars) PBMC from five normal adults. $^{*}P < .05$ compared with uninfected cells. &$P < .05$ compared with $CD8^+$ HSV-infected cells. \$$P < .05$ compared with $CD4^+$ HSV-infected cells. #$P < .05$ compared with $CD45RA^+$ HSV-infected cells (Wilcoxon signed rank test).

lymphocytes from adults. The percentage of cells that expressed IL-2 and TNF-α among HSV-1-infected $CD3^+$, $CD4^+$, $CD8^+$, and $CD45RA^+$ lymphocytes from CBMC was significantly greater than among uninfected cells (Fig. 3). Intracellular IFN-γ was not detected in $CD3^+$, $CD4^+$, $CD8^+$, and $CD45RA^+$ among uninfected CBMC after culture with PMA plus monensin. HSV-1 infection did not enhance intracellular IFN-γ levels in the presence of monensin.

Levels of Extracellular IL-2, IFN-γ, and TNF-α in the Supernatant From Uninfected and HSV-1-Infected Cells

The concentration of IL-2, IFN-γ and TNF-α in the supernatant from uninfected and HSV-1-infected PBMC without simulation was at a less than detectable level (data not shown). The concentration of IL-2, IFN-γ, and TNF-α in the supernatant from HSV-1-infected PBMC was greater than that of uninfected PBMC when stimulated with PMA plus ionomycin. The concentration of IL-2 and TNF-α in the supernatant from HSV-1-infected CBMC was greater than that of uninfected CBMC when stimulated with PMA plus ionomycin. There was no significant difference in the amount of IFN-γ in the supernatant from uninfected and HSV-1-infected CBMC (Table I).

Percentage of the Cells Expressing of HSV-1-Antigen Positive Cells

HSV-1-infected PBMC and CBMC were stained with FITC-labeled-anti-HSV-1 antibody 24 hr after stimulation of PMA plus ionomycin. The mean percentage of HSV-1 antigen positive cells among HSV-1-infected PBMC and CBMC were 12% and 45%, respectively.

DISCUSSION

The detection of intracellular cytokines by flow cytometry has been described previously [Anderson et al., 1988; Jung et al., 1993; Prussin and Metcalfe, 1995]. FACS permeabilizing solution is used for the permeabilization of lymphocyte membranes prior to intracellular immunofluorescence staining of antigens, cytokines, and other proteins with MoAbs. Lymphocyte surface antigen was stained with PerCP-conjugated or FITC- or PE-conjugated MoAbs and then stained intracellular cytokines with FITC- or PE-conjugated anti-cytokines antibodies. Thus, the procedures permitted the simultaneous detection of intracellular cytokines and membrane antigens by three-color flow cytometry analysis. Stimulation with PHA did not induce intracellular IL-2, IFN-γ, and TNF-α in either the presence or absence of monensin. It has been demonstrated that the activation of protein kinase C and an increase of intracellular Ca^{2+} are required for IL-2 production [Isakov et al., 1987]. PMA plus ionomycin was used in this study to induce cytokines.

HSV-1 enhanced significantly the intracellular levels of IL-2, IFN-γ, and TNF-α in $CD3^+$ T lymphocytes from normal adults when stimulated with PMA plus ionomycin in the presence of monensin. In adult PBMC, the level of intracellular IL-2 in HSV-1-infected $CD4^+$ lymphocytes exceeded that in HSV-1-infected $CD8^+$ lymphocytes and the level of intracellular IFN-γ in HSV-1-infected $CD8^+$ and $CD45RO^+$ lymphocytes exceeded that in HSV-1-infected $CD4^+$ and $CD45RA^+$ lymphocytes. It has been reported that IL-2 is the dominant cytokine produced by $CD4^+$ lymphocytes, whereas IFN-γ is produced mainly by $CD8^+$ lymphocytes [Mosmann, 1991]. The mechanism of the increase of intra-

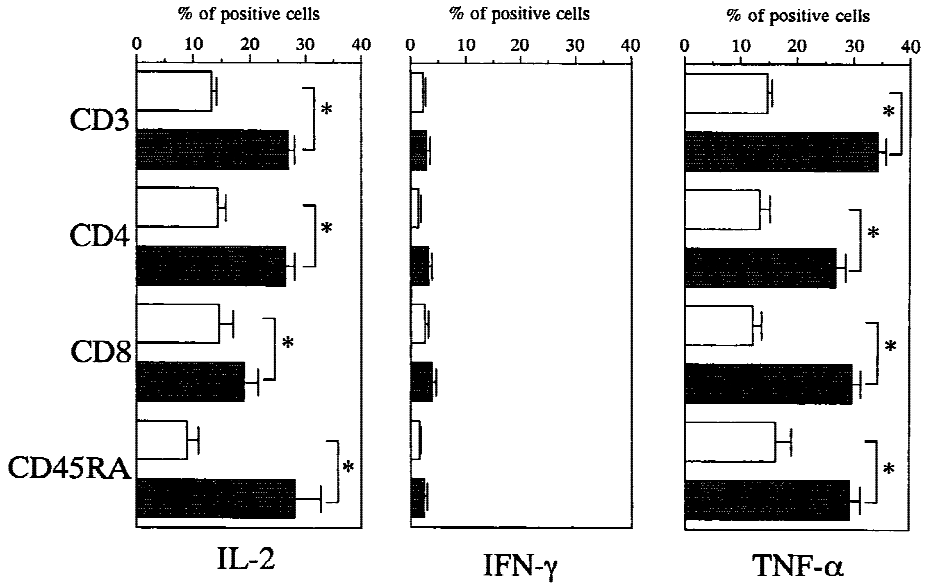

Fig. 3. Expression of intracellular IL-2, IFN-γ and TNF-α in each lymphocyte subpopulation from cord blood mononuclear cells (CBMC). Data represent mean percentages of cells that expressed IL-2, IFN-γ, and TNF-α in HSV-1-infected (shaded bars) and uninfected (open bars) PBMC from five newborns. $P < .05$ compared with uninfected cells (Wilcoxon signed rank test).

TABLE I. Extracellular Levels of IL-2, IFN-γ, and TNF-α in HSV-1-Infected and Uninfected PBMC and CBMC

	IL-2 (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)
HSV-1-infected PBMC	8964	7862	5569
Uninfected PBMC	7455	5155	2752
HSV-1-infected CBMC	7629	364	3569
Uninfected CBMC	4862	352	2153

PBMC, peripheral blood mononuclear cells; CBMC, cord blood mononuclear cells.
HSV-1-infected and uninfected PBMC and CBMC were cultured with phorbol 12-myristate 13-acetate (PMA) plus ionomycin for 24 h and the supernatant was collected. The amounts of IL-2, IFN-γ, and TNF-α in the supernatant were assayed by enzyme-linked immunosorbent assay (ELISA). One representative data from three different experiments was shown.

cellular cytokines in HSV-1-infected cells is unclear. The intracellular levels of IL-2, IFN-γ, and TNF-α in $CD3^+$ lymphocytes did not change when PBMC were treated with inactivated HSV-1 and cultured with PMA plus ionomycin in the presence or absence of monensin (data not shown), indicating that live virus is required to enhance intracellular levels of cytokines. A possible mechanism would be that HSV-1 directly stimulates the production of IL-2, IFN-γ, and TNF-α. Gosselin et al. [1992] have investigated the effects of EBV, HHV-6, and HSV infections on cytokine synthesis and reported that HHV-6 had a much more potent effect on the production of TNF-α protein than HSV-1, whereas larger amounts of TNF-α transcripts were detected in HSV-1-infected cells than in HHV-6 infected cells. It has been reported that infectious HSV-1 stimulates IL-1 and TNF-α gene transcription of mouse peritoneal macrophages [Sprecher and Becker, 1992]. The human cytomegalovirus (HCMV) immediate early gene (IE2) product up-regulates IL-2 and IL-2 receptor gene transcription [Geist et al., 1991]. Like cytomegalovirus, unknown in HSV-1 gene may code cytokine-regulatory proteins and the HSV gene product may bind to the enhancer regions of cytokines. Another possibility is that HSV-1 infection inhibits cells proliferation and results in reduction of degradation of cytokines or a reduced consumption of intracellular cytokines. We investigated the mRNA level of IL-2, IFN-γ, and TNF-α in uninfected and HSV-1-infected cells by competitive reverse transcription-polymerase chain reaction. There was no significant difference in mRNA of IL-2, IFN-γ, and TNF-α between HSV-1-infected and uninfected PBMC (data not shown). Further studies on the steady state level of cytokines are needed to clarify the mechanisms. It was not determined whether intracytokine levels were increased in HSV-1-infected or uninfected cells. Further analysis of intracytokine and HSV-1 gene products at the single cell level is needed.

The effect of HSV-1 on intracellular levels of cytokines of adult lymphocytes was compared with that of cord blood. HSV-1 enhanced the intracellular levels of IL-2 and TNF-α in HSV-1-infected $CD3^+$ T lymphocytes of cord blood by stimulation with PMA plus ionomycin. However, significant levels of IFN-γ were detected in neither HSV-1 infected nor uninfected cells. Low production of IFN-γ, TNF-α, and IL-4 by stimulated CBMC has been reported [Müller et al., 1996; Sautois et al., 1997]. The mechanisms of impaired production of IFN-γ by cord blood have been reported [Lewis et al., 1986; von Freeden et al., 1991]. von Freeden et al. [1991] reported that the defect of IFN-γ might be due to its pretranslational level. English et al. [1988] reported low levels of IFN-γ mRNA in stimulated cord blood. Wakasugi et al. [1985] reported that impaired IFN-γ production could be the result of increased cell sensi-

tivity to the suppressive effects of PGE-1 and not to defect in IL-2 production. Finally, Kruse et al. [1993] suggested that the absence of $CD4^+/CD45RO^+$ memory T cells could be partly responsible for the low IFN-γ production. IFN-γ plays an important role in regulating and mediating cellular immune response. Activation of macrophages, NK cells, and T lymphocytes was observed after IFN-γ treatment [Arenzana-Seisdedos and Virelizier, 1983; Lindahl et al., 1972; Gidlund et al., 1978]. However, the mechanism by which infection with HSV-1 failed to enhance intracellular levels of IFN-γ in cord blood is unclear. Deficient IFN-γ production by cord blood may explain the susceptibility of neonates to HSV infection.

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